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EVALUATION OF COMMERCIALLY PREPARED VACCINES FOR EXPERIMENTALLY--ETC(U)
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→ significantly less illness than unvaccinated controls when challenged with virulent virus 30 days after i.m. immunization with 200 CCA units of whole virus or 400 CCA units of split virus given either once or twice (at 30-day intervals). Equal protection was observed in all monkeys, despite the absence of serum HAI antibody in some monkeys after vaccination. Anamnestic reactions were observed only in monkeys vaccinated with whole virus. The possible roles of various immune factors and antineuraminidase antibody are discussed.

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(6) Evaluation of Commercially Prepared Vaccines for
Experimentally Induced Type A/NJ Virus Influenza Infections in Mice and
Squirrel Monkeys

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(9) *Interim report*

From The U.S. Army Medical Research Institute of Infectious Diseases

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Running head: Protection of Mice and Monkeys

We wish to thank Dr. F. Ennis, Bureau of Biologics, for providing seed stocks of the New Jersey and X-53 recombinant strains of virus and the whole-virus vaccines; and Dr. B. Rueben of Wyeth Laboratories for the split-virus vaccine. We also wish to acknowledge the assistance of G. Higbee and D. Oland for statistical analysis.

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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ABSTRACT

Mice and squirrel monkeys were vaccinated and subsequently challenged at selected times in order to evaluate the immunoprophylactic value of vaccines against influenza virus type A/NJ. Mice were challenged with virulent, homologous virus at either 17 or 60 days after vaccination with 80 CCA units of whole virus vaccine. Vaccinated mice showed minimum lesions and virus in lung tissue, and had lower lung weights than unvaccinated controls. These mice had serum HAI titers of >1:400, but only traces of antibody were found in lung washes. Vaccinated squirrel monkeys had significantly less illness than unvaccinated controls when challenged with virulent virus 30 days after i.m. immunization with 200 CCA units of whole virus or 400 CCA units of split virus given either once or twice (at 30-day intervals). Equal protection was observed in all monkeys despite the absence of serum HAI antibody in some monkeys after vaccination. Anamnestic reactions were observed only in monkeys vaccinated with whole virus. The possible roles of various immune factors and antineuraminidase antibody are discussed.

Most reports concerning the national swine influenza immunization program have dealt with clinical investigation of vaccine efficacy and reactogenicity [1] of human patients. Because the virulence of the New Jersey strain of virus is unknown, experimental inoculation of humans with it has not been attempted in the United States, although Beare and Craig have inoculated volunteers in England [2]. Therefore, vaccine efficacy has been evaluated on a basis of titer of serum hemagglutination-inhibiting antibody rather than protection.

Because our laboratory has containment facilities to limit accidental spread of virus and because we have recently developed models for influenza virus infection in squirrel monkeys [3,4], we have carried out evaluations of several of the commercially prepared vaccines that have been developed for the national swine influenza program.

In this report we present our findings in experimentally-infected mice and squirrel monkeys together with evidence for a lack of correlation between serum HAI antibody titer and protection.

Materials and Methods

Virus preparation and assay. The techniques employed to produce cultures of influenza virus type A/New Jersey/8/76 (Hsw1 N1) for routine use have been described previously [4].

The virus was adapted to mice by intranasal instillation of 0.05 ml of a 10^{-3} dilution of sixth-passage suspension. After 3 to 4 days the lungs were removed aseptically, homogenized in 3.0 ml of HIB, and 0.05 ml inoculated intranasally into additional mice. After a total of nine passages a working stock was prepared by embryonated egg inoculation. The allantoic fluid was collected and stored in 5-ml aliquots at -60 C. The titer of this material was $10^{7.7}$ EID₅₀/ml.

X-53, recombinant virus [5], grown and stored in the same manner as the virulent virus, was used as the antigen for serum HAI determinations. Antigenic identity of the hemagglutinin was confirmed with reference standard materials supplied by the Center for Disease Control (CDC) using CDC procedures [6].

Experimental animals. White Swiss female mice (Crl:COBS CD1[ICR]BR) obtained from the "Sendai-free" Montreal colony of Charles River Laboratories were used. The mice, ranging in age from 6 to 9 weeks, were housed in ventilated safety cabinets. They were allowed free access to commercial mouse pellets and water. Although preliminary investigations showed that the mortality of mice of this age after challenge with the New Jersey strain of virus was less than that in younger animals, the length of time required for vaccine evaluation precluded the use of young mice.

The squirrel monkeys (Saimiri sciureus) used in these studies were obtained from commercial sources. They were males and ranged in weight from 600 to 900 g. Housing and feeding procedures have been described [4].

Virulent virus challenge. Mice, under light anesthesia, were challenged by instilling 0.05 ml of viral suspension, containing 10^6 EID₅₀, into the nares. Monkeys were challenged by intratracheal instillation of 1.0 ml containing 10^7 EID₅₀ of virus.

Clinical and laboratory studies. Because the results of preliminary experiments indicated that the mortality rate in intranasally-challenged mice was low (<25%), the evaluation of response to vaccination was based upon the frequency of isolation of virus and the degree of pathological change in the lungs. The latter was evidenced by weight of the lungs and the extent of gross lesions. The scoring procedure for measuring lung lesions was adapted by Scott and Sydiskis [7] from a technique developed by Fazekas de St. Groth and Donnelley [8]. A score of 4 was given for 100% consolidation, 3 for 75%, 2 for 50%, 1 for 25%, and 0 for normal lungs.

The method of evaluating the clinical response of monkeys to intratracheal instillation of virus has been described [4]. The illness scores derived from the various groups of monkeys were analyzed by analysis of variance and the method of least significant difference.

Experimental plan. Two commercial monovalent vaccines were tested

in the course of these experiments: a whole virus product, manufactured by Merck, Sharpe & Dohme, Inc., containing 400 CCA units/0.5 ml dose; and a sub-unit (split virus) vaccine manufactured by Wyeth Laboratories that also contained 400 CCA units/0.5 ml dose. Adjustment of dosages was based upon label claims of the manufacturers. No additional potency estimates were made.

For preliminary investigations in mice, only the whole-virus vaccine was employed because limited quantities of the split-virus product were available. Ninety mice were each injected intraperitoneally with 0.1 ml of vaccine containing 80 CCA units. At 17 days after vaccination, selected mice were bled from the orbital sinus, killed, bronchoalveolar washings [7] were obtained for antibody determinations, and about one-half of the remaining mice were challenged with 10^6 EID₅₀ of virulent virus. At the same time, sham-vaccinated (saline) control mice as well as mice that had been inoculated with virulent virus 17 days earlier (prior-illness controls) were also challenged. Fifteen mice from each group were killed 7 days later for determination of the presence of virus and lesions in the lungs as well as for determination of weight of the lungs.

Sixty days after vaccination, 15 mice from each group were bled and then killed for subsequent bronchoalveolar washings. The remaining mice were challenged with virulent virus and killed 7 days later to estimate the extent of lesions and virus in the lungs. No prior-illness controls survived for challenge at this time because of a higher than anticipated death rate.

A total of 65 squirrel monkeys were used for evaluations in primates. Serum HAI titers were determined prior to vaccination, 30 days after vaccination (one day prior to challenge), and 7 and 14 days after challenge with virulent virus. The protocol for vaccination is given in table 1. The rationale for the combination of vaccines was based upon the report of Laver and Webster [9]. In the present study, their approach was modified by employing intact homologous virus in a single suspension. All monkeys were observed for 96 hr after vaccination for adverse reactions. Thirty days after the last vaccination, all monkeys were challenged by intratracheal inoculation of 10^7 EID₅₀ of virulent virus. In addition, 15 monkeys that had recovered from illness caused by virulent virus at least 30 days earlier, were challenged as prior-illness controls. Three additional nonvaccinated monkeys were sham-infected with sterile normal allantoic fluid. The clinical response of all monkeys was then followed for 7 days after challenge.

Results

Preliminary experiments with mice. Mice were challenged with virulent virus at 17 and 60 days after vaccination. The 17-day period was chosen because prior experience with influenza virus (serotype H3N2) in this laboratory indicated that peak serum HAI titers would be achieved then; the 60-day period was selected to determine whether protection decreased with time. Results, presented in table 2, indicated that the whole-virus vaccine stimulated significant levels of serum antibody and afforded virtually complete protection as indicated by virus titer and pathologic changes in the lungs. When bronchoalveolar wash specimens were concentrated approximately 10-fold by ultrafiltration and tested for HAI antibody, low HAI titers (1:2) were detected in the prior-illness control mice, and only traces were found in vaccinated mice.

Experiments with squirrel monkeys. After preliminary results demonstrated that the whole-virus vaccine effectively protected mice, investigations were initiated in monkeys.

One to two degrees of fever that lasted from 24-48 hr were the only reactions to vaccination that were seen. There was no anorexia, lethargy or localized reaction at the site of injection. Serum HAI titers were determined at selected intervals prior to and after vaccination and also 7 and 14 days after virulent-virus challenge. These titers are given in figure 1. Since the lowest titer detectable was 1:10, negative sera were assigned an arbitrary titer of 1:5 for purposes of calculating geometric means. None of the vaccination techniques had stimulated the production of significant levels of antibody at the time of virulent virus challenge.

Analysis of the data revealed that of the four groups of vaccinated monkeys, only the HAI titer of the whole-virus group differed significantly from that of the placebo controls ($P < 0.01$) 25 days after vaccination. After challenge the HAI titers of the prior-illness controls, as well as those of the whole virus and combined vaccine groups increased rapidly, suggestive of an anamnestic reaction. In contrast, monkeys vaccinated with either one or two injections of split-virus vaccine reacted to challenge much like the placebo controls, suggesting that these monkeys had little or no memory for the HA antigen. Although two of the monkeys in each of the two split-virus vaccinated groups had titers of $\geq 1:160$ 14 days after challenge, the titers of the two groups did not differ significantly from those of the placebo controls.

The serological data suggested that small amounts of whole virus were necessary for the production of serum HAI antibody. However, the illness scores among the various groups of monkeys after challenge were not consistent with the HAI antibody titrations. The illness scores are summarized in table 3. All methods of vaccination significantly reduced the severity of disease caused by virulent virus ($P < 0.001$). The sham controls manifested only a slight transient response to intratracheal instillation of normal allantoic fluid. Further evidence of lack of relationship between serum HAI titers and illness scores was obtained when coefficients of correlation (r) were calculated for the linear regression of these two variables. The values of r ranged from -0.22 to +0.42.

Although all immunizations significantly reduced illness scores, it was of interest to determine whether there was a significant reduction in virus shedding. The effect of vaccination on virus shedding was evaluated by analyzing the number of days that virus could be isolated by pharyngeal swabbing of the various groups of monkeys. Only the monkeys that received two injections of split-virus vaccine shed virus for a significantly shorter period than the unvaccinated controls ($P < 0.001$) (table 4). The prior-illness controls, however, shed virus for the shortest period.

Discussion

The major objective of these studies was to determine whether commercially produced whole-virus and split-virus (sub-unit) influenza vaccines effectively protect experimental animals against respiratory challenge with the virulent New Jersey strain (swine) of influenza virus. Our data show that mice, vaccinated with 80 CCA units of whole-virus vaccine, were completely protected from infection and illness following intranasal instillation of the New Jersey strain of influenza virus either 17 or 60 days later. The data obtained with squirrel monkeys showed that both vaccines provided partial protection, but neither of them completely prevented illness, and only one vaccination regimen significantly reduced virus shedding. The combination of vaccines did not appear to offer any advantage. Perhaps the most surprising observation was that of a lack of correlation between serum HAI titers and protection in monkeys; indeed, squirrel monkeys usually did not form detectable amounts of serum HAI antibody after vaccination. Antibody levels of vaccinated monkeys after subsequent intratracheal inoculation with virulent virus varied with the type of vaccine; animals that were vaccinated with even small amounts of whole virus developed HAI antibody at a much greater rate than those receiving split virus.

Several ancillary questions have been emphasized by these data: What is the role of antineuraminidase antibody in swine influenza virus infection; what part is played by secretory antibody and by cell-mediated immune factors; and how can we explain the fact that only partial

protection was provided by prior infection? The role of antineuraminidase antibody is especially important because the vaccines employed were reported to be deficient in neuraminidase and only infrequent and low-level responses to it were observed in human volunteers [1]. Neuraminidase has been shown to limit replication of virus in mice, but not the initial infection, and thus should prevent transmission of infection [10-12]. Thus, the monkeys that had previously been infected with virulent virus would be expected to shed less virus after challenge than the vaccinated animals because they were the only monkeys to receive significant amounts of neuraminidase. Also, the monkeys that were immunized twice with split-unit vaccine might be expected to have higher neuraminidase titers than any of the other vaccinated monkeys if even small amounts of neuraminidase were present in this product.

A role for cell-mediated factors in immunity to influenza has recently been discussed by Virelizier et al. [13] who showed that specific immunological recognition and memory may be mediated by T lymphocytes. This observation is of particular interest because all monkeys vaccinated with whole virus had a memory for the Hsw1 antigen, whereas most of those that received split virus did not. Whether other cell-mediated factors mediated by T lymphocytes were active in these monkeys is unknown. Analysis of lower respiratory secretions of squirrel monkeys for antibody was not attempted because of the possibility of exacerbating illness with the lung lavage techniques required to obtain such secretions.

The failure of prior-infection to provide complete protection against

virulent virus challenge may best be explained by the relatively large dose of virus instilled. Dose-response experiments would be necessary to resolve this point.

Finally, the question of the adequacy of the experimental models must be addressed. Mice have been employed for hundreds of investigations with influenza virus, monkeys in a limited number. Since the pathogenesis of the New Jersey strain of influenza virus in humans is unknown there are no means by which severity of infection or efficacy of vaccines in mice and man or monkeys and man can be compared. It is interesting that Beare and Craig's observation of mild illness after intranasal instillation in humans with the New Jersey strain of virus [2] is paralleled by our similar observation in both mice and monkeys.

The serological reaction of squirrel monkeys to split-virus vaccines containing the Hsw1 antigen appears to be much the same as that of human children [1]. The partial protection afforded monkeys by this product suggests that humans may be similarly protected. Since this vaccine is less reactogenic for humans than the whole-virus vaccine, it may be the product of choice. This vaccine, however, has the disadvantage of failing to stimulate the accepted index of immunity in the host, increased HAI antibody titer.

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Table 1. Experimental design for determination of the efficacy of selected vaccines against influenza A/New Jersey/8/76 virus.

Treatment	No. monkeys	Dose of vaccine inoculated (CCA units)
Nonvaccinated sham-infected	3	0
Placebo-vaccinated	19	0
Prior-illness controls	15	0
Split-virus vaccine (1 inoculation)	7	400
Split-virus vaccine (2 inoculations at a 30-day interval)	7	400 (each inoculation)
Whole-virus vaccine	7	200
Combination vaccine (in 1 inoculum)	7	400 split-virus 25 whole-virus

Table 2. Resistance to type A/NJ influenza virus infections by intraperitoneally-vaccinated mice

Treatment (n=15)	Mean serum HAI titer at challenge	Value of indicated lung parameter at 7 days		
		Viru isolation (no. pos./5)	Lesion score and frequency	Weight (mg)
I. Mice challenged 17 days after vaccination.				
HIB (virus control)	0	5	2.1 (10/10)	307
Prior-illness control	113	0*	0.8 (6/10)	268
Whole virus (80 CCA)	463	0*	0.0 (0/10)***	202**
II. Mice challenged 60 days after vaccination.				
HIB (virus control)	0	5	1.6 (10/10)	301
Prior-illness control	905	N.D.	N.D.	N.D.
Whole virus (80 CCA)	499	1	0.0 (0/10)***	206***

Note: Titors are expressed as reciprocals.

Statistical significance (tested against virus control):

$$\begin{array}{ll} * & P < 0.05 \\ ** & \frac{P}{P} < 0.025 \\ *** & \frac{P}{P} < 0.005 \end{array}$$

Table 3. Mean 7-day illness score of vaccinated squirrel monkeys challenged with virulent NJ strain (swine) of influenza virus.

Treatment	Illness Scores*
Placebo controls	42.5 \pm 2.2
Noninfected sham inoculated	4.7 \pm 0.4**
Prior-illness controls	17.0 \pm 1.8**
Split-virus vaccine (1 injection)	24.9 \pm 1.9**
Split-virus vaccine (2 injections)	26.9 \pm 2.5**
Whole-virus vaccine	26.0 \pm 2.4**
Combination vaccine	29.9 \pm 2.1**

* Mean \pm SEM.

** $P \leq 0.001$.

Table 4. Virus shedding by vaccinated monkeys.

Treatment	Mean days virus shedding*
Placebo control	6.5 \pm 0.3
Prior-illness control	2.7 \pm 0.3**
Split-virus vaccine (1 injection)	5.7 \pm 0.6
Split-virus vaccine (2 injections)	3.7 \pm 0.6**
Whole-virus vaccine	5.6 \pm 0.4
Combined vaccine	6.0 \pm 0.4

* Mean \pm SEM.

** $P \leq 0.001$.

Figure Caption

Figure 1. Serum HAI titers of squirrel monkeys prior to and after virulent virus challenge. WPV - whole-virus product vaccine, SVP - split-virus product vaccines (I - one vaccination, II - two vaccinations at 30-day intervals), combined - combined vaccine.

HAI RESPONSE TO VACCINATION AND CHALLENGE

